

## Muscarinic and nicotinic receptors raise intracellular $\text{Ca}^{2+}$ levels in rat carotid body type I cells

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1. The effects of cholinergic agonists upon intracellular free  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ) have been studied in enzymically isolated rat carotid body single type I cells, using indo-1.
2. Acetylcholine (ACh) dose-dependently increased  $[\text{Ca}^{2+}]_i$  in 55% of cells studied ( $\text{EC}_{50} = 13 \mu\text{M}$ ). These  $[\text{Ca}^{2+}]_i$  rises were partially inhibited by atropine or mecamylamine.
3. Specific nicotinic and muscarinic agonists also elevated  $[\text{Ca}^{2+}]_i$  in a dose-dependent manner (nicotine,  $\text{EC}_{50} = 15 \mu\text{M}$ ; methacholine,  $\text{EC}_{50} = 20 \mu\text{M}$ ).
4. While the majority of the ACh-sensitive cells responded to both classes of cholinergic agonist, 29% responded exclusively to nicotinic stimulation and 9% responded exclusively to muscarinic stimulation.
5. In the presence of nicotinic agonists,  $\text{Ca}_i^{2+}$  responses were transient. In the presence of muscarinic agonists,  $\text{Ca}_i^{2+}$  responses consisted of an initial rise, which then declined to a lower plateau level.
6. Nicotinic responses were rapidly abolished in  $\text{Ca}^{2+}$ -free medium, suggesting that they are dependent on  $\text{Ca}^{2+}$  influx.
7. The plateau component of the muscarinic-activated response was also abolished in  $\text{Ca}^{2+}$ -free conditions. The rapid initial  $[\text{Ca}^{2+}]_i$  rise, however, could still be evoked after several minutes in  $\text{Ca}^{2+}$ -free medium. Muscarine also increased  $\text{Mn}^{2+}$  quenching of intracellular fura-2 fluorescence. These data suggest that the full muscarinic response depends on both  $\text{Ca}^{2+}$  release from intracellular stores and  $\text{Ca}_o^{2+}$  influx.
8. The results indicate that, in rat carotid body type I cells, both nicotinic and muscarinic acetylcholine receptors increase  $[\text{Ca}^{2+}]_i$ , but achieve this via different mechanisms. ACh may therefore play a role in carotid body function by modulating  $\text{Ca}_i^{2+}$  in the chemosensory type I cells.

The carotid body is a sensor of arterial  $P_{\text{O}_2}$ ,  $P_{\text{CO}_2}$  and pH. The primary sensory element is thought to be the type I cell which is in synaptic contact with the afferent terminals of the carotid sinus nerve (CSN). Type I cells contain a wide range of neurotransmitters including catecholamines and acetylcholine, which are secreted actively in response to chemostimuli. The role of these transmitters, however, is at present unclear. Acetylcholine (ACh) was originally proposed to act as the excitatory neurotransmitter between type I cells and sensory nerve endings on account of its potent stimulatory effects on CSN discharge in the cat (see Fidone & González, 1986, for review). Many observations, however, contest this hypothesis. Firstly, the effects of ACh on neural discharge depend upon the species. For example, in the rabbit ACh induces initial depression followed by a secondary increase in chemosensory discharge (Monti-Bloch & Eyzaguirre, 1980). Secondly, and more importantly,

cholinergic antagonists fail to block the normal CSN response to hypoxia (Nishi & Eyzaguirre, 1971; Sampson, 1971; McQueen, 1977; cf. Fitzgerald & Shirahata, 1994). In addition, autoradiographic studies have failed so far to detect cholinergic receptors on the afferent terminals of the carotid sinus nerve.

In contrast with the lack of compelling evidence for a role for ACh as an excitatory transmitter between type I cells and sensory nerve endings, there is growing evidence that ACh may act directly upon the type I cell. Both nicotinic and muscarinic acetylcholine receptors have been found to be abundant in type I and type II cells (Dinger, Gonzalez, Yoshizaki & Fidone, 1985; Dinger, Hirano & Fidone, 1986). Moreover, nicotinic agonists evoke inward currents in isolated type I cells (Wyatt & Peers 1993). In addition, preliminary evidence shows that cholinergic agonists raise  $[\text{Ca}^{2+}]_i$  (Biscoe, Duchon, Eisner, O'Neill & Valdeolmillos,

1989; Peers, Wyatt & Buckler, 1994) and evoke  $^{45}\text{Ca}^{2+}$  influx (Pietruschka, 1985) in rabbit and rat type I cells. The mechanisms responsible for this increase of  $[\text{Ca}^{2+}]_i$  and  $^{45}\text{Ca}^{2+}$  influx are at present unknown.

The effects of cholinergic agonists upon presynaptic (type I cell)  $[\text{Ca}^{2+}]_i$  are potentially of considerable interest since the chemotransduction of physiological stimuli such as hypoxia and acidosis are also mediated via changes in type I cell  $[\text{Ca}^{2+}]_i$  (Biscoe & Duchen, 1990; Buckler & Vaughan-Jones, 1994a, b; Ureña, Fernandez-Chacon, Benot, Alvarez de Toledo & López-Barneo, 1994). Therefore the effects of cholinergic receptor stimulation upon intracellular calcium in isolated rat type I cells have been studied using fluorescence microscopy. Both nicotinic and muscarinic receptors are involved in mediating an abrupt rise of  $[\text{Ca}^{2+}]_i$  in response to acetylcholine, albeit through different mechanisms. This presynaptic  $[\text{Ca}^{2+}]_i$  response to acetylcholine might help to explain the exquisite sensitivity of the intact organ to exogenously applied ACh (Monti-Bloch & Eyzaguirre, 1980). In addition, we discuss possible physiological roles for the presynaptic actions of ACh.

A preliminary report of this work has been published (Dasso, Buckler & Vaughan-Jones, 1996)

## METHODS

### Carotid body type I cell isolation

Sprague-Dawley rats aged 11–16 days were anaesthetized with 4% halothane and their carotid bodies excised and stored in ice-cold phosphate-buffered saline pre-equilibrated with 100% oxygen. At the end of the dissection procedure rats were killed by decapitation. The carotid bodies were incubated at 37 °C in a low- $\text{Ca}^{2+}$  (65  $\mu\text{M}$ ) phosphate-buffered saline containing 0.4 mg  $\text{ml}^{-1}$  collagenase and 0.2 mg  $\text{ml}^{-1}$  trypsin. After 20 min the carotid bodies were teased apart with forceps and incubated for another 5 min in the same medium. The pieces were gently triturated with a fire-polished Pasteur pipette and the cell suspension centrifuged at 180  $g$  for 5 min. Cells were resuspended in a culture medium consisting of bicarbonate-buffered Ham's F-12 medium containing insulin (84 U  $\text{l}^{-1}$ ), penicillin (100 i.u.  $\text{ml}^{-1}$ ), streptomycin (100  $\mu\text{g}$   $\text{ml}^{-1}$ ), L-glutamine (1 mM) and 10% (v/v) fetal calf serum. The dispersed cells were then plated onto poly-D-lysine-coated 6 mm diameter glass coverslips and kept in culture medium at 37 °C in an atmosphere of 5%  $\text{CO}_2$  and 95% air for 4–8 h until use.

### $[\text{Ca}^{2+}]_i$ measurements in single cells

$[\text{Ca}^{2+}]_i$  was measured using indo-1 essentially as previously described (Buckler & Vaughan-Jones, 1993). Cells were loaded with indo-1 by incubation with 2.5  $\mu\text{M}$  indo-1 acetoxymethyl ester for 1 h at room temperature (19–24 °C) in an atmosphere of 5%  $\text{CO}_2$  in air. After dye loading, the coverslips with the cells attached were transferred to Petri dishes containing the culture medium indicated above, without added indo-1, and kept in the dark until use. Coverslips were then transferred to a perfusion chamber mounted on the stage of an inverted microscope (Nikon Diaphot) with a  $\times 40$  quartz oil-immersion objective operating in epifluorescence mode. The volume of buffer in the chamber was typically 100  $\mu\text{l}$ . Cells were continuously superfused with a standard bicarbonate-buffered Tyrode solution or a test solution fed by a peristaltic pump (Minipuls 3, Gilson, Anachem, Luton, Beds, UK) at a flow rate of

1.5 or 2  $\text{ml min}^{-1}$ . A two-way valve was used to change solutions perfusing the chamber. Upon switching solutions, the entire volume of the chamber was exchanged in 5–8 s. All experiments were performed at 35–37 °C.

Type I cells were identified by their appearance and their response to an anoxic stimulus (5%  $\text{CO}_2$  and 95%  $\text{N}_2$  plus 1 mM sodium dithionite). A large, rapid rise (5–15 s) in  $[\text{Ca}^{2+}]_i$ , indicating substantial  $\text{Ca}_o^{2+}$  influx due to opening of voltage-operated  $\text{Ca}^{2+}$  channels (Buckler & Vaughan-Jones, 1994a), identified a type I cell (Fig. 1A). The resting  $[\text{Ca}^{2+}]_i$  in type I cells was found to be  $74 \pm 2$  nM ( $n = 167$ ). The anoxia-evoked increase in  $[\text{Ca}^{2+}]_i$  was  $1200 \pm 55$  nM ( $n = 136$ ). An acute exposure to anoxia did not affect the magnitude of the  $[\text{Ca}^{2+}]_i$  elevations evoked by subsequent challenges with 300  $\mu\text{M}$  methacholine ( $n = 13$ ,  $P = 0.6$ ) or 100  $\mu\text{M}$  nicotine ( $n = 12$ ,  $P = 0.87$ ), indicating that it was not detrimental to cholinergic responses (not shown).

Indo-1 fluorescence was determined at 405 and 495 nm with excitation at 340 nm. Excitation light (100 W xenon lamp) was passed through neutral density filters (0.39% transmission) to minimize photobleaching during experiments. Fluorescence was detected by two trialkali photomultiplier tubes (Thorn EMI) cooled to  $-20$  °C, whose output was digitized, and the ratio of intensities was calculated by a microcomputer throughout the experiments. Data were integrated over 500 ms intervals. The conversion of fluorescence ratios into  $[\text{Ca}^{2+}]_i$  was accomplished by *in situ* calibration of a separate group of cells, essentially as described by Thomas & Delaville (1991). Briefly, cells loaded with indo-1 were incubated in a  $\text{Ca}^{2+}$ -free Tyrode solution, containing 5 mM EGTA and 10  $\mu\text{M}$  ionomycin, for 30 min at room temperature. The coverslip with the cells attached was then perfused with the same solution, at 37 °C, except that the concentration of ionomycin was now 5  $\mu\text{M}$ . Cells were then superfused with a Tyrode solution containing 10 mM  $\text{CaCl}_2$  and 5  $\mu\text{M}$  ionomycin. Finally, cells were superfused with a Tyrode solution containing 5 mM  $\text{MnCl}_2$  and 5  $\mu\text{M}$  ionomycin to obtain an estimate of autofluorescence levels at each wavelength.  $[\text{Ca}^{2+}]_i$  was calculated according to the equation:

$$[\text{Ca}^{2+}]_i = K_d (S_{f2}/S_{b2})(R - R_{\min})/(R_{\max} - R),$$

where  $R_{\min}$  is the ratio value for the unbound form of indo-1,  $R_{\max}$  is the ratio value for the bound form of indo-1,  $S_{f2}/S_{b2}$  is the ratio of fluorescence values for free and bound indo-1 at the wavelength that monitors the free indicator (495 nm), and  $K_d$  is the dissociation constant for indo-1/ $\text{Ca}^{2+}$  (250 nM) (Gryniewicz, Poenie & Tsien, 1985).

### Assessment of $\text{Ca}^{2+}$ influx

Changes in the rate of  $\text{Ca}^{2+}$  influx were determined using the method of  $\text{Mn}^{2+}$  quench of fura-2 fluorescence (Merritt, Jacob & Hallam, 1989).  $\text{Mn}^{2+}$  can substitute for  $\text{Ca}^{2+}$  in influx pathways and its entry can be monitored as it binds with high affinity to fura-2 and thereby quenches its fluorescence. Cells were loaded with fura-2 by incubation in a 2.5  $\mu\text{M}$  solution of fura-2 acetoxymethyl ester for 30 min. Fluorescence was excited alternately at 380 and 360 nm (isosbestic point) and measured at 510 nm. Quenching of the 360 nm fluorescence signal reflects  $\text{Mn}^{2+}$  entry from the extracellular medium into the cytoplasm. The signal recorded with this excitation wavelength is  $\text{Ca}^{2+}$  independent.

### Analysis of results

Changes in  $[\text{Ca}^{2+}]_i$  were calculated as the difference between the peak  $[\text{Ca}^{2+}]_i$  value elicited by exposure to a pharmacological agent and the mean resting  $[\text{Ca}^{2+}]_i$  value determined prior to the exposure. All experimental protocols were repeated at least four times with cells from different preparations. Data were expressed as

means  $\pm$  S.E.M. Statistical significance of the results was assessed by Student's two-tailed paired *t* test. Curves were analysed using the iterative program GraphPAD InPlot 4.01 (GraphPad Software, Inc., San Diego, CA, USA). Data for each individual dose-response experiment were fitted to a four-parameter equation:

$$Y = (B - A) / [1 + EC_{50}/X]^{n_H}] + A,$$

where *Y* is the  $[Ca^{2+}]_i$  elevation, *B* is the maximal  $[Ca^{2+}]_i$  elevation evoked by saturating concentrations of agonist, *A* is the  $[Ca^{2+}]_i$  elevation at zero agonist concentration, *X* is the concentration of agonist,  $EC_{50}$  is the concentration of agonist causing 50% of the maximal response, and  $n_H$  is the Hill coefficient. The individual  $EC_{50}$  values obtained for each experiment were averaged and are expressed in the text as means  $\pm$  S.E.M.

### Solutions

The standard Tyrode solution contained (mM): 117 NaCl, 4.5 KCl, 22  $NaHCO_3$ , 1  $MgCl_2$ , 2.5  $CaCl_2$  and 11 D-glucose, equilibrated with 5%  $CO_2$  and 95% air; pH 7.4–7.45 at 37 °C.  $Ca^{2+}$ -free medium was prepared by omitting  $CaCl_2$  from the Tyrode solution and adding 1 mM EGTA. High- $K^+$  Tyrode solution (50 mM  $K^+$ ) was prepared by replacing NaCl with an equimolar concentration of KCl. Anoxia was achieved by equilibrating solutions with 5%  $CO_2$  and 95%  $N_2$  and adding 1 mM  $Na_2S_2O_4$ . Drugs were dissolved in

the above Tyrode solution. All solutions were prepared freshly each day, with the exception of the stock solutions of mecamylamine and muscarine, which were aliquoted and frozen at  $-20^\circ C$ .

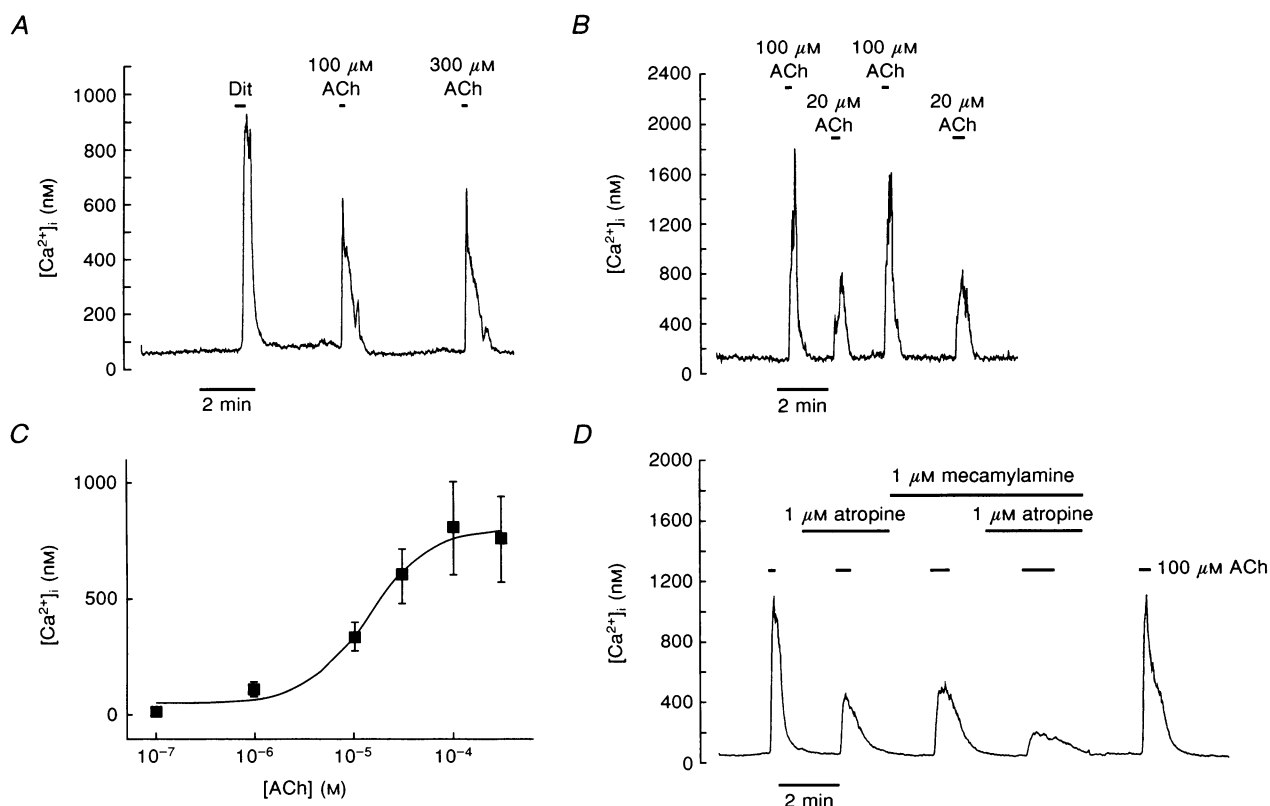
### Materials

Acetylcholine, nicotine, muscarine, methacholine, oxotremorine, atropine, mecamylamine, poly-D-lysine, EGTA, sodium dithionite, manganese chloride, trypsin Type III, Ham's F-12 medium, phosphate-buffered salines, insulin, L-glutamine and penicillin/streptomycin were from Sigma. Oxotremorine M was from Research Biochemicals Inc. Fetal calf serum (heat inactivated) was from Gibco. Indo-1 AM, fura-2 AM and ionomycin were from Calbiochem. Collagenase Type 1 was from Worthington (Freehold, NJ, USA). All other chemicals were of the highest grade commercially available.

## RESULTS

### Studies in 2.5 mM $Ca^{2+}$ -containing medium

Type I cells responded to the non-specific endogenous cholinergic agonist ACh (0.1–300  $\mu M$ ) with a rapid elevation of  $[Ca^{2+}]_i$  (Fig. 1*A* and *B*).  $[Ca^{2+}]_i$  responses were concentration dependent. Figure 1*C* shows the amplitude of  $[Ca^{2+}]_i$  responses as a function of ACh concentration.



**Figure 1.** Effect of ACh on  $[Ca^{2+}]_i$  in single type I cells in  $Ca^{2+}$ -containing medium

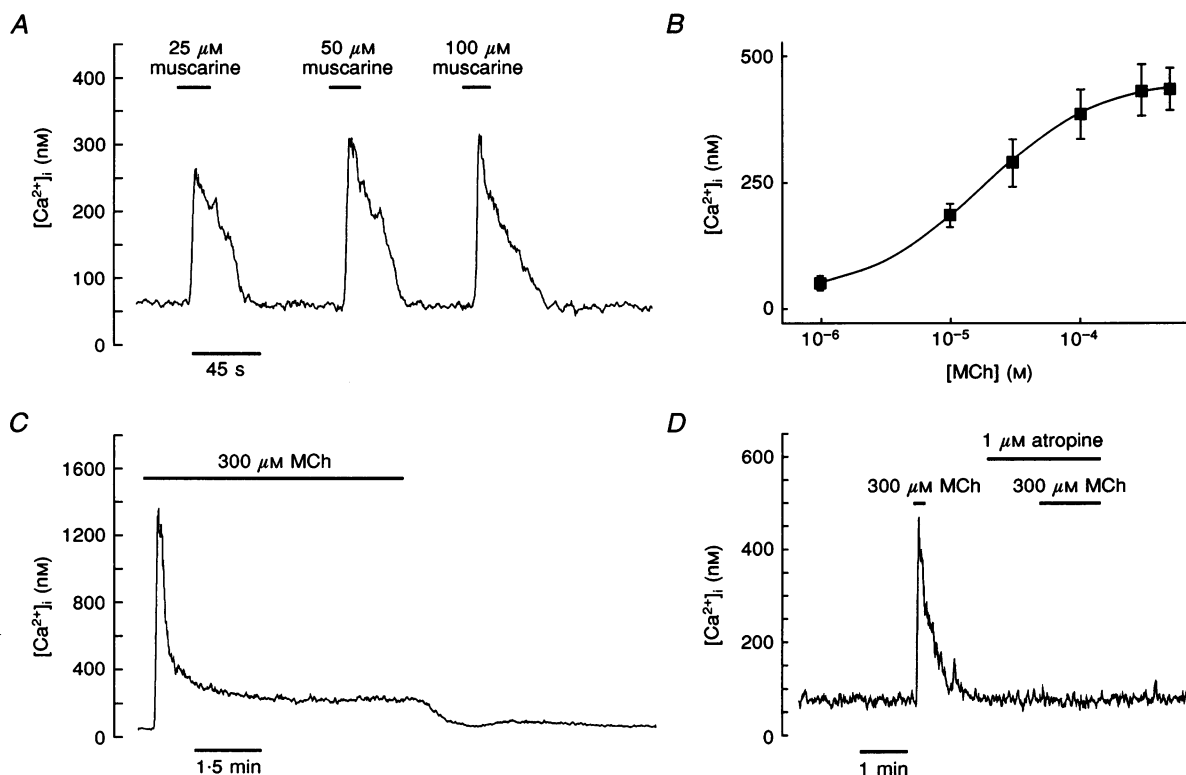
*A* and *B*, type I cells were loaded with indo-1 and then challenged with ACh, as indicated by event markers. *A* also shows the effect of anoxia, i.e. superfusion with medium equilibrated with 5%  $CO_2$  and 95%  $N_2$  in the presence of 1 mM sodium dithionite (Dit). *C*, concentration-response relationship between ACh and  $[Ca^{2+}]_i$ . Results are expressed as means  $\pm$  S.E.M. Data at each concentration were obtained from twelve cells. Data were fitted to a four-parameter logistic equation (see Methods);  $r^2 = 0.969 \pm 0.005$ . *D*, effect of muscarinic and nicotinic antagonists on the ACh-induced  $[Ca^{2+}]_i$  response. Type I cells loaded with indo-1 were challenged with ACh (100  $\mu M$ ) in the presence of the antagonists atropine (1  $\mu M$ ), mecamylamine (1  $\mu M$ ) or both, as indicated.

Maximal responses were obtained with  $100\ \mu\text{M}$  ACh. The 50% maximal effective ACh concentration ( $\text{EC}_{50}$ ) was  $13 \pm 3\ \mu\text{M}$  ( $n = 12$ ). The average peak value of the elevation in  $[\text{Ca}^{2+}]_i$  evoked by  $100\ \mu\text{M}$  ACh was  $798 \pm 76\ \text{nM}$  above basal ( $n = 89$ ). However, not all type I cells responded to stimulation with  $100\ \mu\text{M}$  ACh. Of 182 cells tested, 82 (45%) failed to show an increase in  $[\text{Ca}^{2+}]_i$  upon challenge with  $100\ \mu\text{M}$  ACh. The responses evoked by  $100\ \mu\text{M}$  ACh were partially abolished by the muscarinic antagonist atropine ( $1\ \mu\text{M}$ ;  $61 \pm 10\%$  inhibition,  $n = 16$ ) or the nicotinic antagonist mecamylamine ( $1\ \mu\text{M}$ ;  $65 \pm 5\%$  inhibition,  $n = 16$ ). The simultaneous presence of both antagonists inhibited the elevations of  $[\text{Ca}^{2+}]_i$  elicited by ACh by  $88 \pm 3\%$  ( $n = 16$ ; Fig. 1*D*).

Having established that type I cells respond to activation of cholinergic receptors with elevations of  $[\text{Ca}^{2+}]_i$ , experiments were conducted using well-defined specific muscarinic and nicotinic agonists. Cells responding to the muscarinic agonist methacholine ( $1$ – $500\ \mu\text{M}$ ) did so with a rapid increase in  $[\text{Ca}^{2+}]_i$  (e.g. Fig. 2*C*). The concentration–response relationship for methacholine is shown in Fig. 2*B*.

$\text{EC}_{50}$  was  $20 \pm 6\ \mu\text{M}$  ( $n = 9$ ). When cells were exposed to maximal concentrations of methacholine ( $300\ \mu\text{M}$ ),  $[\text{Ca}^{2+}]_i$  rose to  $672 \pm 87\ \text{nM}$  ( $n = 39$ ) above basal. Other muscarinic agonists muscarine ( $10$ – $100\ \mu\text{M}$ ;  $n = 59$ ; Fig. 2*A*), oxotremorine ( $100\ \mu\text{M}$ ;  $n = 77$ ) and oxotremorine M ( $100\ \mu\text{M}$ ;  $n = 7$ ; Ringdahl, 1988) also evoked  $[\text{Ca}^{2+}]_i$  rises.

In most experiments described in this paper, agonists were applied for short periods of time (15–45 s). In order to characterize the temporal profile of the muscarinic response in more detail, some cells were exposed to maximal concentrations of muscarinic agonists for longer periods of time. When type I cells were challenged with  $300\ \mu\text{M}$  methacholine for longer than 1 min, the  $[\text{Ca}^{2+}]_i$  response was clearly biphasic. In these experimental conditions  $[\text{Ca}^{2+}]_i$  levels peaked rapidly ( $634 \pm 215\ \text{nM}$  above basal,  $n = 6$ ) and then declined to a sustained and elevated plateau value ( $108 \pm 19\ \text{nM}$ , i.e.  $17 \pm 3\%$  of the peak value; range 12–54%,  $P < 0.003$  vs. basal,  $n = 6$ ; basal was  $89 \pm 14\ \text{nM}$ ) while methacholine was present in the superfusion medium. Only when methacholine was washed away did  $[\text{Ca}^{2+}]_i$  return to resting levels (Fig. 2*C*).



**Figure 2.** Effect of muscarinic agonists on  $[\text{Ca}^{2+}]_i$  in  $\text{Ca}^{2+}$ -containing medium

*A*, type I cells were challenged with the muscarinic agonist, muscarine, as indicated. *B*, concentration–response relationship between methacholine (MCh) and  $\text{Ca}^{2+}$ . Results are expressed as means  $\pm$  s.e.m. Data at each concentration were obtained from nine cells. Data were fitted to a four-parameter logistic equation (see Methods);  $r^2 = 0.95 \pm 0.01$ . *C*, when cells were exposed to MCh for longer than 1 min,  $[\text{Ca}^{2+}]_i$  peaked and then declined to a sustained and elevated plateau value in the presence of MCh. Removal of MCh caused  $[\text{Ca}^{2+}]_i$  to return to resting levels. *D*, effects of atropine ( $1\ \mu\text{M}$ ) on the  $[\text{Ca}^{2+}]_i$  response to  $300\ \mu\text{M}$  MCh.

Addition of nicotine (0.1–500  $\mu\text{M}$ ) led to elevations of  $[\text{Ca}^{2+}]_i$  (Fig. 3A). The  $\text{EC}_{50}$  for nicotine was  $15 \pm 3 \mu\text{M}$  ( $n = 10$ ; Fig. 3B). Nicotine (100  $\mu\text{M}$ ) elicited maximal elevations of  $[\text{Ca}^{2+}]_i$  ( $701 \pm 80 \text{ nM}$ ,  $n = 42$ ). When cells were exposed to nicotine for longer periods of time ( $> 1 \text{ min}$ ),  $[\text{Ca}^{2+}]_i$  rose ( $683 \pm 161 \text{ nM}$ ,  $n = 18$ ) and then declined (Fig. 3C). In some experiments, a slightly elevated plateau phase could be discerned, but in most cells  $[\text{Ca}^{2+}]_i$  returned in about 1 min to resting levels, which were indistinguishable from those observed before application of nicotine. On average, the resting  $[\text{Ca}^{2+}]_i$  in nicotine was elevated by  $1.1 \pm 0.01\%$  of the peak increase in  $[\text{Ca}^{2+}]_i$  ( $n = 16$ ) and was not significantly different from control levels in the absence of nicotine ( $P > 0.1$ ). In two cells out of the eighteen tested, after the nicotine-evoked  $[\text{Ca}^{2+}]_i$  elevation subsided, a second transient elevation of similar magnitude was observed (a plateau value could not be estimated in these cells and consequently they were not considered for the basal–plateau comparisons).

Figures 2 and 3 show the effects of adding specific antagonists prior to the application of muscarinic or nicotinic agonists. Responses to methacholine were inhibited  $96 \pm 3\%$  by the muscarinic antagonist atropine at a concentration of 1  $\mu\text{M}$  ( $n = 12$ ; Fig. 2D). The nicotinic antagonist mecamylamine at a concentration of 1  $\mu\text{M}$  completely prevented the appearance of nicotinic responses ( $n = 7$ ; Fig. 3D). Furthermore, 1  $\mu\text{M}$  mecamylamine had no

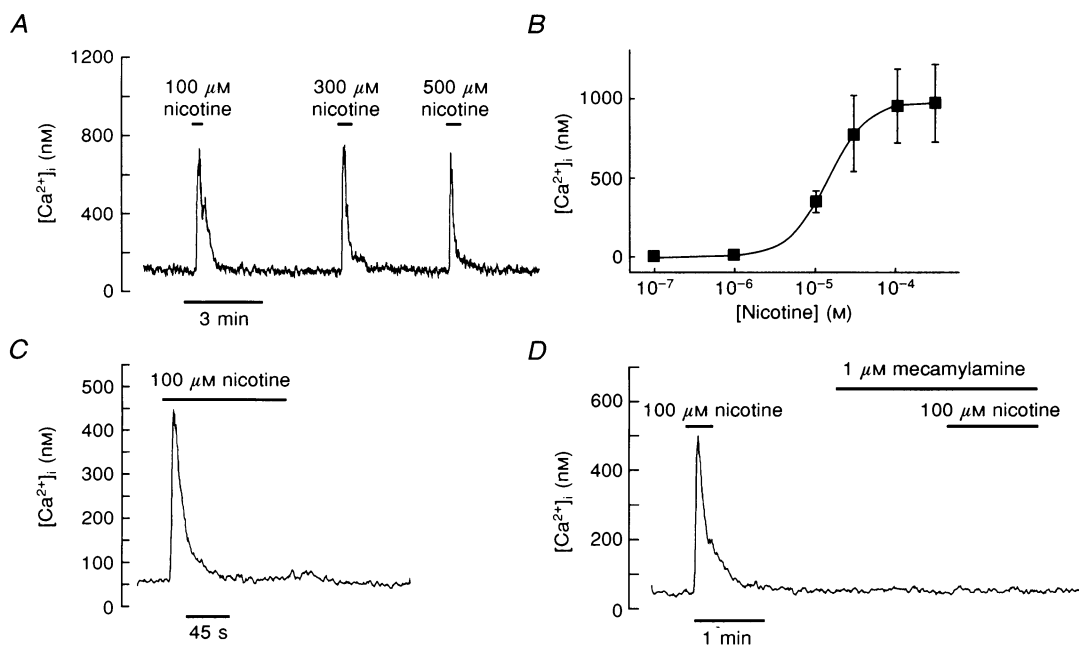
effect on MCh-evoked responses ( $P = 0.97$ ,  $n = 4$ ), strongly suggesting that nicotinic receptors are not activated by MCh at the concentrations used.

Responses of different cells displayed variation in amplitude, duration, pattern of the elevation (including, in some cells, oscillatory behaviour), sensitivity to agonist concentration and lag time before onset of the response following exposure to the different cholinergic agonists; on the other hand, for a given cell, the response to each agonist was usually reproducible and did not decline with repetitive challenges (see Figs 1, 2 and 3).

In order to investigate the distribution of muscarinic and nicotinic responses in type I cells, we exposed a number of ACh-responding cells to methacholine (300  $\mu\text{M}$ ) and nicotine (100  $\mu\text{M}$ ; Fig. 4A). Of the thirty-five cells investigated, twenty-two responded to both agonists (63%), while some responded only to methacholine ( $n = 3$ ; 9%) or only to nicotine ( $n = 10$ ; 29%). The responses to either class of agonist showed a large variation in amplitude. No correlation was observed between muscarinic and nicotinic responses (Fig. 4B).

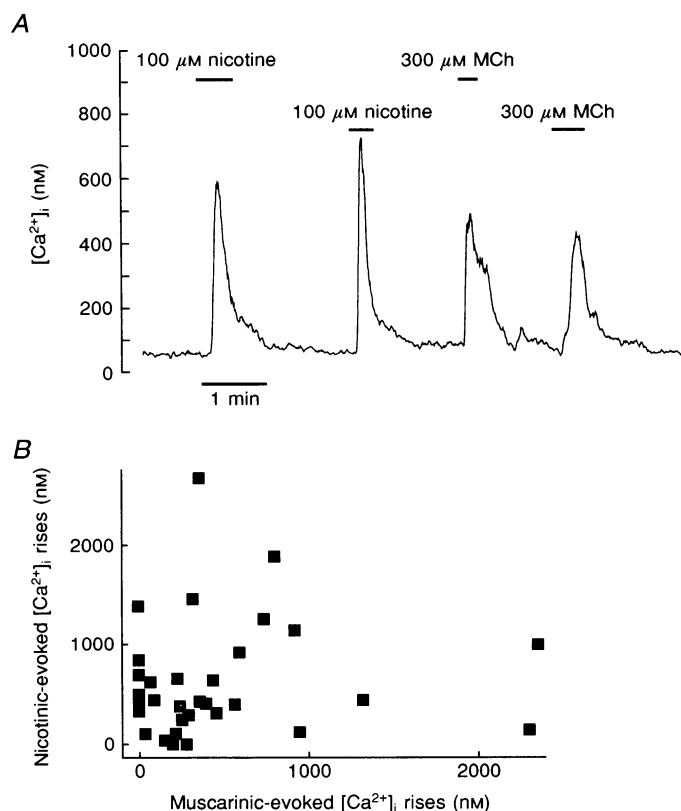
#### Studies in $\text{Ca}^{2+}$ -free medium

The following experiments examined the source of  $\text{Ca}^{2+}$  implicated in the responses to activation of nicotinic and muscarinic receptors. When cells were exposed to  $\text{Ca}^{2+}$ -free medium, the resting  $[\text{Ca}^{2+}]_i$  was significantly reduced. The



**Figure 3.** Effect of nicotine on  $[\text{Ca}^{2+}]_i$  in  $\text{Ca}^{2+}$ -containing medium

A, cells were exposed to nicotine, as indicated. B, concentration–response relationship between nicotine and  $\text{Ca}^{2+}$ . Results are expressed as means  $\pm$  s.e.m. Data at each concentration were obtained from ten cells. Data were fitted to a four-parameter logistic equation (see Methods);  $r^2 = 0.987 \pm 0.007$ . C, when cells were challenged with nicotine for longer periods of time,  $[\text{Ca}^{2+}]_i$  peaked and returned to basal levels. D, effects of mecamylamine (1  $\mu\text{M}$ ) on the  $[\text{Ca}^{2+}]_i$  response to 100  $\mu\text{M}$  nicotine.

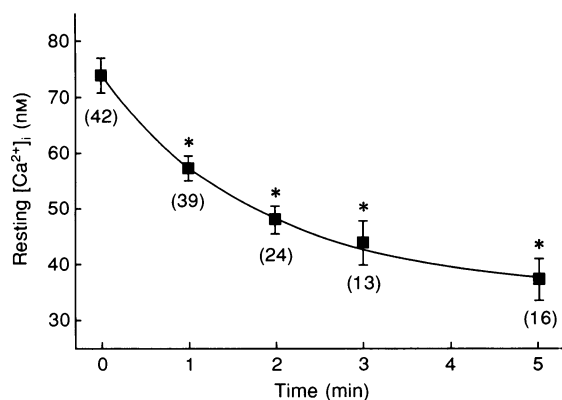


**Figure 4.** Effect of methacholine and nicotine on  $[Ca^{2+}]_i$  in  $Ca^{2+}$ -containing medium

*A*, cells were exposed to 100  $\mu$ M nicotine and 300  $\mu$ M methacholine (MCh), as indicated; the trace illustrates a cell that responded to both classes of cholinergic agonists. *B*, relationship between nicotinic responses and muscarinic responses. ACh-responding type I cells were sequentially challenged with 100  $\mu$ M nicotine and 300  $\mu$ M methacholine.  $[Ca^{2+}]_i$  values are peak levels above basal attained with each agonist. Results shown are from thirty-five cells.

time course of this decline in  $[Ca^{2+}]_i$  could be fitted by a single exponential curve with a half-time ( $t_{1/2}$ ) of 1.3 min (Fig. 5). When  $Ca^{2+}_o$  was reintroduced,  $[Ca^{2+}]_i$  frequently underwent a pronounced transient rebound during which one or more spontaneous spikes could sometimes be observed (Fig. 6*A* and *B*).

In  $Ca^{2+}$ -free medium, addition of muscarinic agonists evoked a transient increase in  $[Ca^{2+}]_i$  (Fig. 6*A*). After 1 min of pre-incubation in  $Ca^{2+}$ -free medium the 300  $\mu$ M MCh-evoked  $[Ca^{2+}]_i$  elevation was  $299 \pm 36$  nM compared with  $629 \pm 81$  nM ( $n = 20$ ) in  $Ca^{2+}$ -containing medium (muscarine in  $Ca^{2+}$ -free medium also evoked a  $[Ca^{2+}]_i$  elevation of



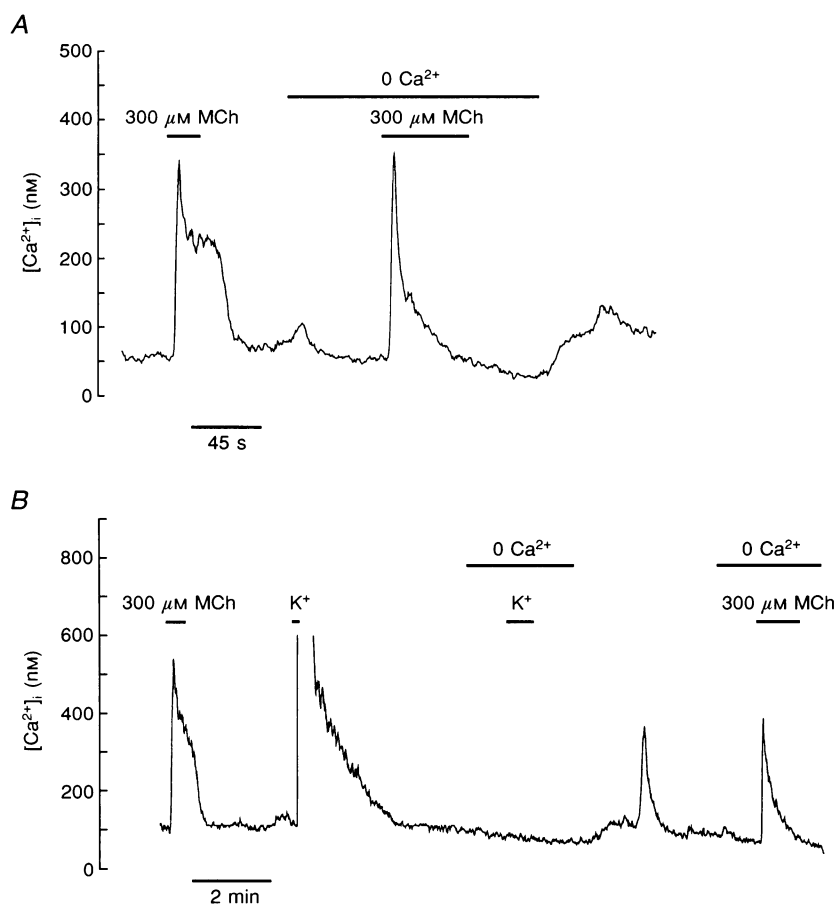
**Figure 5.** Time course of resting  $[Ca^{2+}]_i$  decline in  $Ca^{2+}$ -free medium

Cells were incubated in  $Ca^{2+}$ -free medium (containing 1 mM EGTA) for increasing periods of time. Results are expressed as means  $\pm$  s.e.m. The data were fitted by a single exponential of the form:  $Y = Ae^{-Bt} + C$ , where  $A = 38.6$ ,  $B = 0.54$  and  $C = 35.0$  ( $r^2 = 1.000$ ). The number in parentheses below each point represents the number of cells. \*  $P < 0.0001$  vs. control.

$249 \pm 60$  nM, whilst under control conditions the elevation was  $689 \pm 165$  nM,  $n = 4$ ). In  $\text{Ca}^{2+}$ -free conditions,  $[\text{Ca}^{2+}]_i$  eventually returned to levels that were actually lower than basal in the continued presence of MCh ( $62 \pm 4$  nM; basal =  $66 \pm 4$  nM;  $P < 0.0005$ ,  $n = 14$ ). Figure 6B shows that incubation in  $\text{Ca}^{2+}$ -free medium for the same amount of time (1 min), completely abolished the response to 50 mM  $\text{K}^+$ , thus ruling out the possibility that residual unchelated  $\text{Ca}^{2+}$  in the bath was responsible for the methacholine-evoked  $[\text{Ca}^{2+}]_i$  transients. Taken together, these observations suggest that the initial  $[\text{Ca}^{2+}]_i$  elevation induced by muscarinic agonists is due to release of  $\text{Ca}^{2+}$  from intracellular stores. They further suggest that influx of  $\text{Ca}_o^{2+}$  is necessary to sustain the elevated plateau phase observed after the initial spike. In general, muscarinic agonist-evoked  $[\text{Ca}^{2+}]_i$  transients were of lesser amplitude in  $\text{Ca}^{2+}$ -free medium than in the presence of extracellular  $\text{Ca}^{2+}$ . To gain further insight into the cause of this decline, we studied

methacholine-evoked  $[\text{Ca}^{2+}]_i$  transients after pre-incubation of cells in  $\text{Ca}^{2+}$ -free buffer for varying periods of time.  $[\text{Ca}^{2+}]_i$  transients decreased with increasing pre-incubation time in  $\text{Ca}^{2+}$ -free medium, disappearing completely at 15 min (Fig. 7). Time courses of decline in the amplitude of the  $[\text{Ca}^{2+}]_i$  transients were well fitted by a single exponential function with  $t_{1/2} = 1.2$  min ( $n = 12$ ).

When cells were exposed to nicotine in the absence of  $\text{Ca}_o^{2+}$ , no elevation in  $[\text{Ca}^{2+}]_i$  was observed ( $n = 12$ ), consistent with the notion that the nicotinic acetylcholine receptors mediate  $[\text{Ca}^{2+}]_i$  elevations that are dependent on extracellular  $\text{Ca}^{2+}$  (Sargent, 1993) (Fig. 8). Subsequent superfusion of cells with  $\text{Ca}^{2+}$ -containing medium prompted a ready recovery of the nicotinic response ( $n = 7$ ). The amplitude of the nicotine-evoked responses after reperfusion with  $\text{Ca}^{2+}$ -containing buffer ( $652 \pm 136$  nM,  $n = 6$ ) was not significantly different from the amplitude observed before perfusion with  $\text{Ca}^{2+}$ -free buffer ( $650 \pm 122$  nM,  $P = 0.9$ )



**Figure 6.** Effect of muscarinic agonists on  $[\text{Ca}^{2+}]_i$  in  $\text{Ca}^{2+}$ -free medium

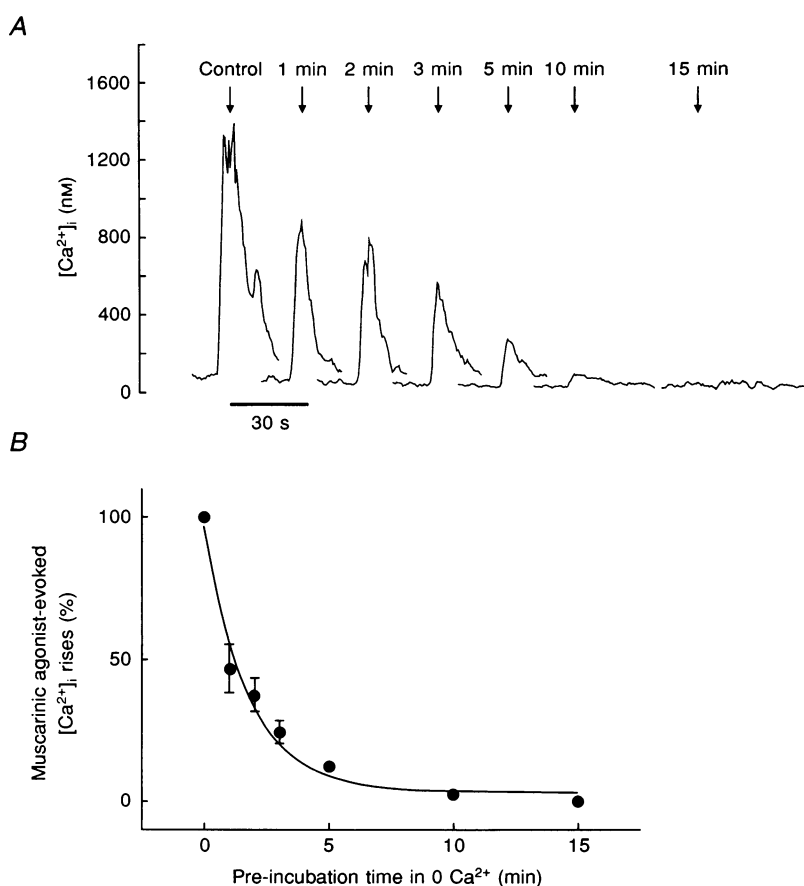
Cells were exposed to 300  $\mu\text{M}$  MCh (A) or 50 mM  $\text{K}^+$  and 300  $\mu\text{M}$  methacholine (MCh) (B), in the presence or absence of extracellular  $\text{Ca}^{2+}$  (containing 1 mM EGTA), as indicated. Note the transient rebound in  $[\text{Ca}^{2+}]_i$  when cells are superfused with  $\text{Ca}^{2+}$ -containing medium after a challenge with  $\text{Ca}^{2+}$ -free medium (A and B). In B a spontaneous  $\text{Ca}^{2+}$  spike can also be observed during the rebound phase. The absence of response evoked by 50 mM  $\text{K}^+$  confirms that the superfusion medium is  $\text{Ca}^{2+}$  free.

### Muscarine-evoked $\text{Ca}^{2+}$ influx

In order to demonstrate more directly the influx component of the muscarinic response, we made use of the method of  $\text{Mn}^{2+}$  quench of fura-2 fluorescence (Merritt *et al.* 1989).  $\text{Ca}^{2+}$  influx into the cytoplasm can be followed by replacing  $\text{Ca}^{2+}$  with  $\text{Mn}^{2+}$  in the extracellular medium and monitoring quenching of the fluorescence caused by excitation of fura-2 at the isosbestic wavelength (360 nm). Muscarine (100  $\mu\text{M}$ ) dramatically augmented the rate of quenching of intracellular fura-2 fluorescence. The time course for the muscarine-evoked fluorescence quench followed first-order kinetics, with rate constant  $k = 0.04 \pm 0.02 \text{ s}^{-1}$  ( $n = 5$ ; Fig. 9). These results strongly suggest that activation of muscarinic receptors stimulates a  $\text{Mn}^{2+}$  entry pathway in type I cells.

### DISCUSSION

This is the first study to examine in detail the effects of cholinergic stimulation on  $[\text{Ca}^{2+}]_i$  signalling in type I cells. We find that type I cells respond to ACh with increases in  $[\text{Ca}^{2+}]_i$  that are a consequence of activation of either muscarinic or nicotinic receptors or both. Indeed, the majority of cholinergic-sensitive cells (63%) responded to both classes of agonist. Nevertheless, a smaller fraction of cells showed  $[\text{Ca}^{2+}]_i$  responses to only muscarinic (9%) or nicotinic (29%) stimulation. Moreover, 45% of all cells tested failed to respond to the non-specific agonist ACh. Biscoe *et al.* (1989) also observed that 61% of rabbit type I cells failed to respond to the mixed cholinergic agonist carbachol. The reason for their observations is not known, but in the present study, all cells responded to an hypoxic

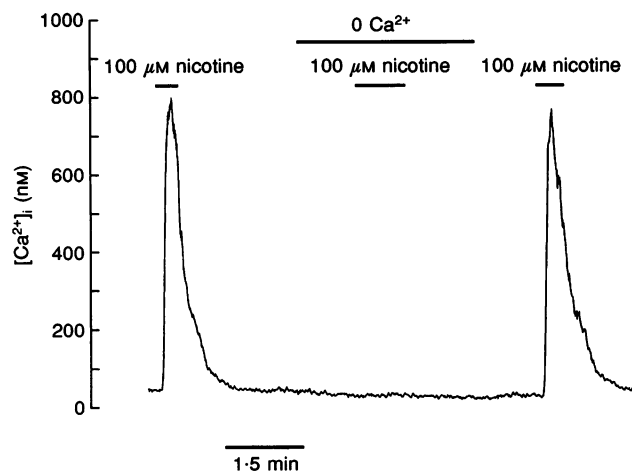


**Figure 7.** Effect of muscarinic agonists on  $[\text{Ca}^{2+}]_i$  in single type I cells after pre-incubation in  $\text{Ca}^{2+}$ -free medium for different periods of time

*A*, cells were challenged with 300  $\mu\text{M}$  methacholine or 100  $\mu\text{M}$  muscarine in  $\text{Ca}^{2+}$ -containing medium ( $t = 0$ ) and subsequently exposed to several cycles that involved: (a) incubation in  $\text{Ca}^{2+}$ -free medium; (b) challenge with 300  $\mu\text{M}$  methacholine or 100  $\mu\text{M}$  muscarine until  $[\text{Ca}^{2+}]_i$  returned to basal levels; (c) superfusion with  $\text{Ca}^{2+}$ -containing medium for 3 min (sufficient time for the resting  $[\text{Ca}^{2+}]_i$  level to stabilize). The figure shows only the responses to 300  $\mu\text{M}$  methacholine from a representative experiment. This protocol was repeated a number of times, varying the pre-incubation times in  $\text{Ca}^{2+}$ -free medium (indicated by the arrows). The times of pre-incubation in  $\text{Ca}^{2+}$ -free medium were randomized. *B*, peak elevations of  $[\text{Ca}^{2+}]_i$  in response to methacholine ( $n = 8$ ) or muscarine ( $n = 4$ ) from twelve independent experiments identical to that described in *A*. Data were fitted to a single exponential function. Results are shown as means  $\pm$  s.e.m. Note that 100  $\mu\text{M}$  muscarine and 300  $\mu\text{M}$  methacholine were found to evoke identical responses ( $n = 5$ ,  $P = 0.2$ ).

**Figure 8.** Effect of nicotine on  $[Ca^{2+}]_i$  in  $Ca^{2+}$ -free medium

Cells were challenged with 100  $\mu M$  nicotine in the presence and absence of extracellular  $Ca^{2+}$ , as indicated. Nicotine failed to increase  $[Ca^{2+}]_i$  in  $Ca^{2+}$ -free conditions. Note the full recovery of the nicotinic response when  $Ca^{2+}_o$  is readmitted.

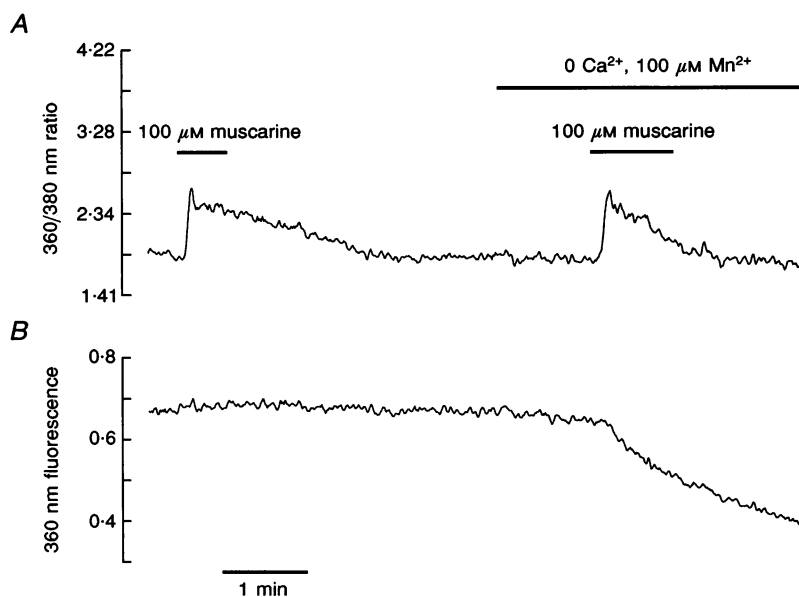


stimulus with a rapid reversible rise of  $[Ca^{2+}]_i$ . This suggests that the cholinergic-insensitive cells were viable and not depolarized, since a reversible hypoxic response requires the membrane potential of the cell in the resting state to be well polarized (e.g. Buckler & Vaughan-Jones, 1994a). We cannot exclude the possibility that, in some cells, cholinergic receptors may have been damaged during enzymic cell isolation. It should be noted, however, that in histological studies of intact rat carotid bodies, only ~50% of type I cells bind the nicotinic antagonist  $\alpha$ -bungarotoxin (Chen & Yates, 1984). The absence of a  $[Ca^{2+}]_i$  response may therefore reflect a genuine physiological phenomenon, i.e. that not all

type I cells possess cholinergic receptors (or alternatively, in the case of muscarinic receptors, that they are either uncoupled or that  $Ca^{2+}$  stores are depleted).

### Nicotinic responses

Nicotinic responses were transient, with  $[Ca^{2+}]_i$  returning to basal levels when still in the presence of the agonist. This desensitization of the nicotine-evoked  $[Ca^{2+}]_i$  response has been described in many other cell types (Marley, 1988), where it is dependent on protein phosphorylation (Hemmings, Nairn, McGuinness, Haganir & Greengard, 1989). The same desensitization phenomenon has also been observed in



**Figure 9.** Effect of muscarine on  $Ca^{2+}$  entry in a single type I cell

Cells were challenged with 100  $\mu M$  muscarine in control medium and then in medium containing 100  $\mu M$   $MnCl_2$  and no added  $Ca^{2+}$ , as indicated. *A*, non-calibrated 360/380 nm fluorescence ratio. Increase in ratio indicates an increase in  $[Ca^{2+}]_i$ . *B*, the 360 nm fluorescence intensity (arbitrary units), which is only sensitive to  $Mn^{2+}$  influx. The first exposure to muscarine evoked an increase in the 360/380 nm fluorescence ratio but no change in fluorescence at 360 nm, thus reflecting an elevation in  $[Ca^{2+}]_i$ . When  $MnCl_2$  was substituted for  $CaCl_2$  in the superfusion medium, a second challenge with muscarine elicited a quench of fluorescence at 360 nm, revealing an increase in the influx of  $Mn^{2+}$ .

electrophysiological experiments, in type I cells, which monitored the membrane current relaxation under whole-cell voltage-clamp conditions (Wyatt & Peers, 1993). In the present work, desensitization was readily reversed: when cells were rechallenged with nicotine after 2–3 min, they responded with  $[Ca^{2+}]_i$  rises of similar magnitude, indicating that the desensitization had subsided.

Nicotinic responses were abolished in the absence of  $Ca^{2+}_o$ ; this result was expected as neuronal nicotinic acetylcholine receptors are known to be ligand-gated cation-selective channels, which mediate  $Ca^{2+}$  influx. In addition, nicotinic receptor activation is also known to depolarize type I cells (Wyatt & Peers, 1993), which could lead to opening of voltage-operated  $Ca^{2+}$  channels. Indeed, carbachol-evoked dopamine release in type I cells has been found to be partially blocked by nitrendipine, suggesting that activation of nicotinic receptors also promotes voltage-gated  $Ca^{2+}$  entry (Shaw, Montague & Pallot, 1989).

### Muscarinic responses

Muscarinic responses were biphasic, characterized by a rapid elevation of  $[Ca^{2+}]_i$  and a subsequent decline to a sustained plateau that lasted until the agonist was removed. Muscarinic agonists were able to evoke a substantial  $[Ca^{2+}]_i$  increase in  $Ca^{2+}$ -free medium, indicating that the  $[Ca^{2+}]_i$  response to muscarinic agonists involves  $Ca^{2+}$  release from internal stores. Indeed, this is the first direct demonstration that type I cells possess such agonist-mobilizable intracellular  $Ca^{2+}$  stores.  $Ca^{2+}$ -free media did, however, modify the response to muscarinic agonists, decreasing  $[Ca^{2+}]_i$  elevations and abolishing the plateau phase observed in  $Ca^{2+}$ -containing medium. These findings suggest that muscarinic agonists may also evoke  $Ca^{2+}$  influx from the external medium, a suggestion strongly supported by the observation that muscarine dramatically increased  $Mn^{2+}$  influx. An increase in  $Ca^{2+}$  permeability may, therefore, play a central role in sustaining the plateau phase of the  $[Ca^{2+}]_i$  response. This muscarine-evoked  $Ca^{2+}$  influx pathway might be triggered by depletion of intracellular stores.  $Ca^{2+}$  release-activated  $Ca^{2+}$  channels (CRACs) have been demonstrated in many non-excitabile cells (Putney, 1990) and also recently in neuroblastoma (Mathes & Thompson, 1994) and cerebellar granule cells (Simpson, Challis & Nahorski, 1995). Additionally, muscarinic receptors might cause cell membrane depolarization, thus evoking  $Ca^{2+}$  influx (Caulfield, 1993). In previous electrophysiological studies, however, muscarinic agonists failed to induce any inward current at  $-70$  mV (Wyatt & Peers, 1993).

The amplitude of the initial  $[Ca^{2+}]_i$  response to muscarinic agonists was found to decline with prolonged exposure to  $Ca^{2+}$ -free medium, indicating a reduction in the amount of agonist-releasable  $Ca^{2+}$ . In principle, this could result from a progressive reduction in the  $Ca^{2+}$  content of the intracellular stores and/or from a  $Ca^{2+}$ -dependent deactivation of receptor coupling mechanisms. The rate of decline of the

muscarine-releasable intracellular  $Ca^{2+}$  in  $Ca^{2+}$ -free medium was surprisingly rapid ( $t_{1/2} = 1.2$  min), such that after only 5–15 min the response to muscarinic agonists was completely abolished. In comparison, run-down of carbachol-evoked  $Ca^{2+}$  release in gastric parietal cells has a  $t_{1/2}$  of  $\sim 30$  min (Negulescu & Machen, 1993). In the type I cell, therefore, agonist-sensitive  $Ca^{2+}$  release would seem to be unusually labile under  $Ca^{2+}_o$ -free conditions. It was also noted that, in  $Ca^{2+}$ -free medium, the time course of decline of agonist-sensitive  $Ca^{2+}$  release was very similar to the time course of the decline in basal  $[Ca^{2+}]_i$  ( $t_{1/2} = 1.3$  min). This observation suggests a causal link between the two processes. The nature of this link is at present unknown, although it is tempting to speculate that store-loading or receptor-dependent  $Ca^{2+}$  mobilization may be critically dependent upon  $[Ca^{2+}]_i$ . Whatever the cause, the decline in agonist-releasable  $Ca^{2+}$  was completely reversed within 3 min of returning the cells to  $Ca^{2+}$ -containing medium.

Muscarinic receptors have been found in other cells to couple to phosphoinositidase C-mediated phosphatidylinositol-4,5-bisphosphate hydrolysis in the plasma membrane, through a pertussis toxin-insensitive G protein, and thus evoke a  $[Ca^{2+}]_i$  rise (Evans, Martin, Hughes & Harden, 1985). This  $Ca^{2+}$  response comprises an initial inositol 1,4-5-trisphosphate ( $IP_3$ )-stimulated release of  $Ca^{2+}$  from intracellular stores that is followed or accompanied by  $Ca^{2+}$  influx across the plasma membrane (Caulfield, 1993). Although agonist-mediated phosphoinositidase C activation or  $IP_3$  formation have not yet been demonstrated in type I cells, this is likely to be the mechanism responsible for the mobilization of intracellular sources of  $Ca^{2+}$  induced by muscarinic agonists. Indeed, it is evident from our experiments that muscarinic agonists increase  $[Ca^{2+}]_i$  in type I cells following a dual mechanism, i.e. mobilization of intracellular  $Ca^{2+}$  stores and influx of extracellular  $Ca^{2+}$ . This dual mechanism is responsible for the biphasic response observed upon prolonged application of muscarinic agonists.

### Other sources of $Ca^{2+}$

In other cell types,  $Ca^{2+}$  influx and  $IP_3$ -mediated  $Ca^{2+}$  release can evoke a secondary release of  $Ca^{2+}$  by a process of calcium-induced calcium release (CICR) (Berridge, 1993). We have previously reported that, in the absence of  $Ca^{2+}_o$ , caffeine evokes a small increase of  $[Ca^{2+}]_i$  in rat type I cells (Buckler & Vaughan-Jones, 1994b). Thus, ryanodine receptors would appear to be present and coupled to intracellular  $Ca^{2+}$  stores. It is therefore conceivable that  $Ca^{2+}$  influx (or indeed  $Ca^{2+}$  release from other functionally distinct stores) might activate CICR in the type I cell. It is also notable that, in bovine chromaffin cells, nicotine and high  $K^+$  can increase  $IP_3$  production through a  $Ca^{2+}$ -dependent activation of phosphoinositidase C (Nakaki, Sasakawa, Yamamoto, & Kato, 1987; Eberhard & Holz, 1988). Whether any of these mechanisms play a significant, secondary, role in the calcium responses mediated by either nicotinic or muscarinic stimulation remains to be determined.

### Role of $[Ca^{2+}]_i$ elevations evoked by ACh

The chemotransduction of physiological stimuli in type I cells is currently thought to involve a rapid rise in  $[Ca^{2+}]_i$  through voltage-operated  $Ca^{2+}$  channels (Buckler & Vaughan-Jones, 1994a, b), which promotes neurosecretion (Ureña *et al.* 1994) and thereby excitation of afferent nerve endings. In the light of this, our results strongly suggest that the potent excitatory effects of exogenously applied ACh may also be mediated through changes in type I cell  $[Ca^{2+}]_i$ . The physiological role of ACh, and of type I cell cholinergic receptors is less certain. To date, ACh in the carotid body has only been found in type I cells (Wang, Stensaa, Dinger & Fidone, 1989) whence it would appear to be released during carotid body stimulation (Eyzaguirre & Zapata, 1984). Similarly, cholinergic receptors in the carotid body are principally located on type I and type II cells. No  $\alpha$ -bungarotoxin or quinuclidinyl benzylate binding sites have been detected on the afferent terminals of the carotid sinus nerve (Dinger *et al.* 1985, 1986). On the other hand,  $\alpha$ -bungarotoxin significantly decreases hypoxia-induced dopamine release and chemoreceptor activity in cat carotid bodies, suggesting that activation of nicotinic receptors and type I cell neurosecretion are at least partially coupled (Dinger *et al.* 1985). It is therefore tempting to speculate that ACh may act as a transmitter between type I cells.

It is also notable that a small percentage of type I cells (4–5%) have been reported to be innervated by preganglionic sympathetic fibres (see Fidone & González, 1986). Cholinergic receptors may therefore also be involved in the efferent control of the chemosensory discharge. However, very little is known about the role of this efferent sympathetic pathway.

Finally, it can be hypothesized that the coexistence of both nicotinic and muscarinic acetylcholine receptors in type I cells, which share the same endogenous ligand, but rely on different sources of  $Ca^{2+}$  to increase  $[Ca^{2+}]_i$ , suggests that these cholinergic receptor subtypes may be responsible for the regulation of different  $Ca^{2+}$ -dependent processes in these cells.

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